Growth- and Differentiation-Associated Expression of bcl-2 in B-Chronic Lymphocytic Leukemia Cells

By Marina Schena, Lars-Gunnar Larsson, Daniela Gottardi, Gianluca Gaidano, Mats Carlsson, Kenneth Nilsson, and Federico Caligaris-Cappio

The bcl-2 gene is translocated into the Ig loci in about 80% of human follicular lymphomas and in 10% of B-type chronic lymphocytic leukemias (B-CLL), resulting in a high level of expression. We have compared the expression of bcl-2 transcripts and protein in B-CLL cells in their normal equivalent CD5+ B cells and in normal B-cell populations representative of different in vivo and in vitro stages of activation and proliferation. We report here that bcl-2 was expressed in 11 of 11 cases of CD5+ B-CLL clones, contrasting with the absent expression in normal CD5+ B cells. Activation of 173 and 183 B-CLL cells by phorbol esters (12-0-tetradecanoylphorbol-13-acetate [TPA]) to IgM secretion without concomitant DNA synthesis resulted in a rapid but transient downregulation of bcl-2 expression. In contrast, the reduction of bcl-2 at both the messenger RNA and protein levels was sustained after mitogenic stimulation, suggesting that bcl-2 expression and proliferation are inversely related in these cells. This notion was further supported by immunocytochemical analysis showing that bcl-2 was primarily expressed in small resting lymphocytes and in cells differentiating to the plasma cell stage, but less expressed in Ki67-positive proliferating B blasts. Moreover, it was also supported by the low level of bcl-2 in exponentially growing Epstein-Barr virus-carrying lymphoblastoid and B-CLL cell lines. The regulation of bcl-2 expression in B-CLL resembled that of normal tonsillar follicular B cells, in which a high level of expression was found in resting mantle zone B cells but not in the proliferating germinal center B cells. Based on these findings and the role of bcl-2 in maintaining follicular B cells, in which a high level of expression was found in resting mantle zone B cells but not in the proliferating germinal center B cells, we propose that the phenotype of B-CLL cells corresponds to a mantle zone memory-type B cell.

The bcl-2 GENE codes for a 25-Kd protein located at the inner mitochondrial membrane. The gene is translocated into the heavy chain Ig locus in a high proportion of human lymphomas of follicular center cell phenotype and into the light chain Ig loci in some cases of B-cell chronic lymphocytic leukemia (B-CLL), resulting in a high constitutive expression of bcl-2 messenger RNA (mRNA) and protein. Introduction of the human bcl-2 gene into murine B-cell precursors results in increased survival in the absence of otherwise essential growth factors and high-level expression of bcl-2 confers a selective growth advantage to mature resting B cells. Moreover, it was also supported by the low level of bcl-2 expression in B-CLL resembling that of normal tonsillar follicular B cells, in which a high level of expression was found in resting mantle zone B cells but not in the proliferating germinal center B cells. Based on these findings and the role of bcl-2 in maintaining B-cell memory, we propose that the phenotype of B-CLL cells corresponds to a mantle zone memory-type B cell.

MATERIALS AND METHODS

Cells. CD5+ malignant B lymphocytes were obtained from nine patients with B-CLL (seven males and three females, age 56 to 67 years). According to the Rai's staging system, two patients were in stage 0-I, three in stage II, two in stage III, and two in stage IV. None of the patients had received chemotherapy in the 4 months preceding this study. Peripheral blood lymphocytes (PBL) were separated from the PB of the patients on Ficoll-Hypaque (FH). T lymphocytes were removed by two cycles of rosetting with sheep red blood cells (SRBC) when the proportion of slg-CD3+ cells exceeded 5%.

173 and 183 are well-characterized selected B-CLL clones. Tonsils (two samples) from adults undergoing tonsillectomy after antibiotic treatment were teased with blunt forceps. Cell

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suspensions were washed with RPMI 1840 medium and adhered to plastic for 1 hour. The nonadherent cells were resuspended with SRBC and spun onto FH for 30 minutes. The cells at the interphase (SRBC−), adherent cell-depleted, and, thus, B-cell-enriched lymphocytes were reacted with the murine monoclonal antibody (MoAb) RFA-2 that, within peripheral lymphoid tissues, recognizes all B and T cells with the notable exception of GC B cells (GC-B). RFA-2+ B cells, which correspond to non-GC-B cells, were purified by goat (G) antimouse (M0)-IgG-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway) and recovered by removing magnetic beads after freezing the cells in cool nitrogen. RFA-2+ B cells are usually enriched in mantle (M) zone B lymphocytes and shall be called M-B here. The cells in the pellet (RFA-2+ cells corresponding to GC-B blasts) were frozen and stored in cool nitrogen.

CD5+ normal B lymphocytes were purified from cord blood cells (two samples), as previously described. Cell lines used were the follicular lymphoma cell line Karpas 422, containing a (14;18) involving the bcl-2 locus; the Burkitt’s lymphoma cell line Du15;7 the EBV-immortalized B-CLL cell line Corinna I and the EBV+ lymphoblastoid cell line Corinna I originating from the same patient.* Cell lines used were the follicular lymphoma cell line Karpas 422, containing a (14;18) involving the bcl-2 locus; the Burkitt’s lymphoma cell line Daudi;* the EBV-immortalized B-CLL cell line Corinna I and the EBV+ lymphoblastoid cell line Corinna I originating from the same patient.*

Phenotype. The phenotype of the cells studied was defined by different combinations of antibodies. Rabbit (R) antisera to human IgM, IgG, IgA, IgD, and IgE chains, directly conjugated with tetraethyl-rhodamine isothiocyanate (TRITC; Dakopatts, Glostrup, Denmark; cat. no. R-152, R-148, R-151, R-153, R-154, R-155), were used in direct immunofluorescence (IF). The MoAbs used were CDS (Leu1; Becton Dickinson, Mountain View, CA; cat. no. 6300), CD10 (Becton Dickinson; cat. no. 7500), CD19 (B4; Coulter, Luton, UK; cat. no. 6602683), CD20 (B1; Coulter; cat. no. 660214), CD38 (A 10), CD23 (MHM6; kind gift of Dr John Gordon, Birmingham, UK), CD25 (Becton Dickinson; cat. no. 7640), CD3 (Leu 4; Becton Dickinson; cat. no. 7340), HLA-DR (Becton Dickinson; cat. no. 7360). MoAbs were used in indirect IF and shown by R-anti-MO-Ig fluorescein isothiocyanate (FITC; Dakopatts; cat. no. F 261), as previously described. A Zeiss epifluorescence microscope (Carl Zeiss Inc, London, UK) equipped with a planapochromat 63×/1.4 oil immersion lens was used throughout this study.

Cell cultures and inductions. 173 and 183 B-CLL cells were thawed, washed with RPMI-1640 medium, and cultured at a concentration of 2 × 10^6/mL in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamycin, in the presence of different stimuli. All cell lines used were used in direct immunofluorescence (IF). The MoAbs used were CDS (Leu1; Becton Dickinson, Mountain View, CA; cat. no. 6300), CD10 (Becton Dickinson; cat. no. 7500), CD19 (B4; Coulter, Luton, UK; cat. no. 6602683), CD20 (B1; Coulter; cat. no. 660214), CD38 (A 10), CD23 (MHM6; kind gift of Dr John Gordon, Birmingham, UK), CD25 (Becton Dickinson; cat. no. 7640), CD3 (Leu 4; Becton Dickinson; cat. no. 7340), HLA-DR (Becton Dickinson; cat. no. 7360). MoAbs were used in indirect IF and shown by R-anti-MO-Ig fluorescein isothiocyanate (FITC; Dakopatts; cat. no. F 261), as previously described. A Zeiss epifluorescence microscope (Carl Zeiss Inc, London, UK) equipped with a planapochromat 63×/1.4 oil immersion lens was used throughout this study.

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Induced differentiation was evaluated by morphologic examination of May-Grünewald-Giemsa-stained cytopsin slides and by measuring the amount of secreted IgM using the enzyme-linked immunosorbent assay (ELISA) technique.

Southern blot analysis. High molecular weight (MW) DNA was isolated using standard methods, digested with restriction enzymes, size fractionated in 0.8% agarose gels, and transferred to nitrocellulose filters. Blots were hybridized with 32P-labeled probes, washed in 0.5% sodium dodecyl sulfate (SDS), 2X SSC (1X SSC = 0.15 mol/L NaCl, 0.015 mol/L NaCitrate, pH 7.0) at 65°C, and autoradiographed (Kodak XAR-5; Eastman Kodak, Rochester, NY) at -70°C. The filters were reused with different probes after removal of the previous signal by washing with 0.1X SSC/0.1% SDS at 100°C. The bcl-2 probes used were a 2.8-kb EcoRI-HindIII genomic fragment corresponding to the major breakpoint region (MBR) in the untranslated 3' end of the bcl-2 gene and a 1.6-kb EcoRI-EcoRI fragment corresponding to the 5' end breakpoint region (kind gifts of Dr Y. Tsujimoto, The Wistar Institute of Anatomy and Biology, Philadelphia, PA).

Northern blot analysis. Total cellular RNA was isolated by the lithium chloride/urea procedure. Denatured total RNA samples (15 μg/well) were fractionated on a 1% formaldehyde-containing agarose gel, transferred to a nitrocellulose filter, and hybridized with 32P-labeled probes in 3X SSC, 0.2% SDS, 1X Denhardt’s solution, 100 μg/mL denatured salmon sperm DNA, and 50% formamide, at 42°C for 18 hours. The filters were washed twice under stringent conditions (0.1X SSC, 0.1% SDS) at 54°C and exposed to Kodak X-OMAT films for 1 to 14 days at -80°C. The bcl-2 probe used was the MBR probe. The actin probe was a mouse α-actin cDNA clone, p91.37

Immunocytochemistry. The cells were cytocentrifuged, dried for 30 minutes, and then fixed in acetone for 10 minutes. After three washes in phosphate-buffered saline (PBS), the slides were incubated for 1 hour with specific antibodies. The antibodies used were monoclonal anti-bcl-2 antibodies, diluted 1:10 (kindly provided by Dr David Mason, John Radcliffe Hospital, Headington, UK), and monoclonal anti-Ki67 antibodies (Dakopatts; cat. no. M772), diluted 1:10. R-anti-M Ig antibodies were used as a second layer. The subsequent procedures used Vectastain peroxidase ABC reagents (Vector Laboratories Inc, Burlingame, CA) for the Ki67 staining and APAAP (Dakopatts) for the bcl-2 staining. The peroxidase activity was visualized by 3-aminophenylcarbazol and the alkaline phosphatase activity by Naphthol AS-MX phosphate/Fast blue.

Western blot analysis. Cells were lysed in a buffer containing 25 mmol/L Tris, pH 8, 1% NP40, 0.5% deoxycholate, 144 mmol/L NaCl, 0.1% SDS, 5 mmol/L dithiothreitol (DTT), 1% trysol, 10 mmol/L lodoacetamide, and 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF) at 4°C. After sonication three times for 15 seconds, the lysates were cleared by centrifugation at 17 K for 30 minutes. The extract (200 μg/lane) was boiled for 4 minutes in sample buffer (0.1 mol/L Tris, pH 6.8, 3% SDS, 5% β-mercaptoethanol), run on a 13% SDS/polyacrylamide gel, and subsequently electrotransferred overnight in transfer buffer (25 mmol/L Tris, pH 7.4, 20 mmol/L glycine, 20% methanol, and 0.1% SDS) to a nitrocellulose filter. The filter was preblocked in 2% nonfat milk, 0.05% Tween 20 in PBS for 1 hour before overnight incubation with anti-bcl-2 antibodies diluted 1:50. Alkaline phosphatase-conjugated goat anti-M Ig (Bio-Rad Laboratories, Richmond, CA) diluted 1:3,000 was used as a second layer and the proteins were visualized by 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitro blue tetrazolium (NBT; Bio-Rad).
bcl-2 expression in B-CLL cells

RESULTS

bcl-2 is expressed in B-CLL. B-CLL cells were purified from nine patients with CLL, representative of different clinical stages of the disease. The cells from all patients coexpressed weak surface (s) monoclonal Ig, CD5, and CD19 as evidenced by IF. None of the cases showed bcl-2 rearrangement within the MBR and the 5' end breakpoint region (data not shown). To investigate the expression of bcl-2 mRNA, total RNA prepared from the B-CLL tumors was subjected to Northern blot analysis using a β²P-labeled bcl-2 probe. As shown in Fig 1A, bcl-2 transcripts were observed in all nine of the fresh B-CLL samples analyzed, the high MW 8.5-kb transcript usually being the most abundant. After densitometrical scanning, the amount of bcl-2 mRNA was normalized to the amount of actin mRNA, used as a reference gene, and to the amount of total RNA loaded as visualized by EtBr staining. Some differences in bcl-2 mRNA expression were observed in the different B-CLL samples, but these seemed to be unrelated both to the clinical stage and to the number of white blood cells (WBC).

The expression of bcl-2 is rapidly but transiently downregulated in I73 B-CLL cells after TPA induction. Because bcl-2 has been reported to be induced during activation of normal resting B and T lymphocytes,10,11 we investigated the expression of bcl-2 mRNA in B-CLL cells activated by TPA. We selected for this experiment one B-CLL clone, I73, which responds to TPA by transition from Go to Gl and differentiation into IgM-secreting lymphoblasts/plasma-blasts in the absence of DNA synthesis.27 Northern blot analysis showed that the steady state level of bcl-2 transcripts in I73 was rapidly downregulated in response to TPA. The level decreased within 1 hour, and reached a minimum (35% of control level) at 3 hours after stimulation (Fig 1B), whereas only minimal changes in the amount of actin mRNA were observed. The level of bcl-2 mRNA subsequently increased and was elevated fourfold as compared with the control cells at 12 and 72 hours postinduction. Also, the level of actin mRNA increased at late time points concomitant with the blast transformation, as previously reported.30 The expression of bcl-2 mRNA late after induction was comparable to that observed in Karpas 422 follicular lymphoma cells, carrying a 14;18 bcl-2 translocation.31 No bcl-2 mRNA was detectable in Daudi Burkitt’s lymphoma cells.

The expression of bcl-2 mRNA is reduced after mitogenic stimulation of B-CLL cells. We have previously described that the B-CLL clone I83 is inducible to activation and subsequent differentiation in the absence or presence of DNA synthesis using selected B-cell activators and cytokines.4,25 To further investigate the relationship of bcl-2 expression to activation, proliferation, and differentiation in B-CLL cells, I83 cells were stimulated for 6 days by different inducers according to five different protocols: TPA, SAC + BSF-MP6 + TNF-α, SAC + BSF-MP6 + IL-2, TPA + BSF-MP6 + IL-4, and SAC + BSF-MP6 + IL-2 + IL-4. As shown in Table 1, TPA induced moderate IgM secretion without DNA synthesis; SAC + BSF-MP6 + TNF-α induced low levels of secreted Ig and moderate DNA synthesis; SAC + BSF-MP6 + IL-2 induced moderate Ig secretion and very high DNA synthesis; and TPA + BSF-MP6 + IL-4 induced very high levels of secreted IgM and high DNA synthesis. The addition of IL-4 inhibited both DNA synthesis and Ig secretion induced by SAC + BSF-MP6 + IL-2, as previously reported.25

The expression of bcl-2 mRNA 6 days after induction was evaluated by Northern blot analysis. Figure 1C shows that the reduction of bcl-2 expression was particularly strong in response to SAC + BSF-MP6 + IL-2 exposure, but also after stimulation by TPA + BSF-MP6 + IL-4. Densitometric scanning and normalization to the amount of actin mRNA and to the amount of loaded total RNA suggested approximate fivefold and threefold reductions, respectively. In contrast, only minor reductions in response to TPA and to SAC + BSF-MP6 + TNF-α, and no change after SAC + BSF-MP6 + IL-2 + IL-4 treatment, were observed. The results thus suggest that bcl-2 is primarily reduced after exposure to factors that induce a high DNA synthesis in I83 cells.

The amount of bcl-2 protein is reduced in I83 B-CLL cells during induced proliferation. To investigate whether the changes in bcl-2 mRNA expression in I83 after mitogenic stimulation were reflected at the protein level, cell extracts obtained from stimulated I83 cells were subjected to a Western blot analysis. As shown in Fig 2, the amount of the 25-Kd bcl-2 protein was reduced to approximately 30% in cells stimulated by SAC + BSF-MP6 + IL-2 for 6 days as compared with uninduced cells. The amount of bcl-2 in TPA + BSF-MP6 + IL-4- and TPA-induced cells was reduced to approximately 55% and 70%, respectively, and was thus similar to the results of the Northern blot analysis. Figure 2 also shows that the amount of bcl-2 in untreated I83 B-CLL cells was comparable with that observed in Karpas 422 cells, which contain a 14;18 bcl-2 translocation.

The results indicate that expression of bcl-2 is inversely related to the proliferation of the B-CLL cells. To further address this question, we analyzed the amount of bcl-2 in the two B-cell lines Corinna I and II during exponential growth. Corinna II represents EBV-immortalized B-CLL cells, whereas Corinna I is an EBV-carrying lymphoblastoid cell line established from normal B cells of the same patient.30 As shown in Fig 2, the amount of bcl-2 in cells from these two cell lines was even lower than in SAC + BSF-MP6 + IL-2–stimulated I83 cells. No bcl-2 protein was detectable in Daudi Burkitt’s lymphoma cells.

Expression of bcl-2 at the single cell level. Because stimulation of I83 B-CLL cells according to the various protocols results in the development of a rather homogenous population of small resting B lymphocytes into a heterogeneous population of cells at different stages of activation and differentiation with or without concomitant proliferation,40 we asked whether bcl-2 was preferentially expressed in certain subpopulations of cells after stimulation. In particular, we wanted to study the amount of bcl-2 in the proliferating cells. Therefore, we examined the expression of bcl-2 and the proliferation-associated nuclear antigen Ki67 in individual cells by immunocytochemical double-labeling.
Fig 1. Northern blot analysis of bcl-2 transcripts in B-CLL. Total cellular RNA (15 µg/lane) was analyzed using a 32P-labeled human bcl-2 probe.26 (A) Lanes 1 through 9, different B-CLL cases; lane 10, normal CD5+ B cells. (B) bcl-2 mRNA expression in I73 B-CLL cells after TPA stimulation. Lane 1, untreated I73 cells; lanes 2 through 6, I73 cells stimulated by TPA for 15 minutes, 1 hour, 3 hours, 12 hours, and 72 hours, respectively; lane 7, Karpas 422 follicular lymphoma cells; lane 8, Deudi Burkitt’s lymphoma cells. (C) bcl-2 mRNA expression in stimulated I83 B-CLL cells. Lane 1, untreated I83 cells; lanes 2 through 7, I83 cells induced by TPA for 4 hours (lane 2); by TPA for 6 days (lane 3); by SAC + BSF-MP6 + TNF-α (lane 4); by SAC + BSF-MP6 + IL-2 (lane 5); by SAC + BSF-MP6 + IL-2 + IL-4 (lane 6); and by TPA + BSF-MP6 + IL-4 (lane 7).
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bcl-2 expression, occasional cells, usually with plasmablast morphology, expressed both Ki-67 and bcl-2 at a high level in a substantial part of the Ki-67-negative population, usually resembling B blasts or activated lymphocytes (Fig 3C). Although most Ki-67-positive cells exhibited a low bcl-2 expression, occasional cells, usually with plasmablast morphology, expressed both Ki-67 and bcl-2 at a high level (Fig 3C). After TPA + BSF-MP6 + IL-4 inductions, which result in a pronounced morphologic and functional differentiation of I83 cells, a more complex distribution of bcl-2 and Ki-67 expression was found. As in SAC + BSF-MP6 + IL-2-stimulated cells, bcl-2 was expressed in the small- to medium-sized lymphocytes and generally had low expression in the larger Ki-67-positive B blasts, whereas the expression in middle-sized lymphocytes was more heterogeneous. However, bcl-2 staining was also often intense in the more differentiated TPA + BSF-MP6 + IL-4–induced cells, resembling plasmablasts (Fig 3D). Corinna II cells, representing EBV-immortalized CLL cells, showed a very weak bcl-2 staining (Fig 3E).

Expression of bcl-2 in normal B-cell populations. Because B-CLL cells are CD5+, we first compared the bcl-2 mRNA level in B-CLL with that of normal CD5+ B cells. Isolated cord blood CD5+ B cells were sIgM+, sIgD+, CD19+, CD20+, HLADR+ and, in a small proportion (20% to 30%), CD23+ and CD25+ (data not shown). As shown in Fig 1A, normal CD5+ B cells from cord blood did not express bcl-2 transcripts.

We next analyzed the bcl-2 expression in normal follicular B cells. The purified follicular B cells were separated into M-B cells and GC-B blasts by their differential reactivity with the RFA-2 antibody. GC-B blasts were sIgM+(>90%), sIgD−(<5%), CD38+(>80%), CD10+(40%), whereas the non–GC-M-B cells were sIgM+, sIgD+, CD19+, CD20+, HLADR+, CD10−, CD38−(data not shown). As shown in Fig 4, the GC-B blasts did not express the message for bcl-2. Neither was cytoplasmic bcl-2 staining observed in these cells using the anti–bcl-2 antibody (data not shown). On the contrary, both bcl-2 message (Fig 4) and the cytoplasmic bcl-2 staining (not shown) were observed in the vast majority (>80%) of resting B mantle cells. bcl-2 mRNA was not detected in total tonsil tissue, but was expressed in monocytes. PBL stimulated with phytohemagglutinin (PHA) + TPA were used as a positive control.

DISCUSSION

The observation that bcl-2/Ig transgenic mice develop an expanded compartment of mature B cells that are 99% in the G0 phase of the cell cycle17 led us to investigate the expression of bcl-2 in B-CLL, which is characterized by the relentless accumulation of mature resting B cells.20,21,24 We have compared here the expression of bcl-2 transcripts and protein in B-CLL cells, in normal CD5+ B cells, and in normal B-cell populations representative of different in vivo stages of activation and proliferation. In two selected B-CLL clones,27 the expression of bcl-2 mRNA and protein has been evaluated also after in vitro treatment with different protocols of activation to growth and/or differentiation. The results show: (1) that malignant CD5+ B-CLL cells express high levels of bcl-2 mRNA and protein, comparable with the levels observed in cells of the Karpass 422 cell line containing a translocated bcl-2 gene, while in normal CD5+ B cells bcl-2 mRNA cannot be detected (Figs 1 and 2); and (2) that the expression of bcl-2 in B-CLL cells is downregulated by mitogenic stimulation.

In agreement with recently reported data,38,41 all B-CLL cases expressed bcl-2. The level of expression seems unrelated to the clinical stage of the patients and does not reflect a rearrangement of bcl-2, which has been shown to be a rare event.42 The fact that the bcl-2 gene is translocated into the Ig light chain loci in approximately 10% of CLL cases studied10 and that B-CLL cells express a higher level of

Table 1. Characteristics of I83 B-CLL Cells Induced to Differentiate With or Without Simultaneous DNA Synthesis

<table>
<thead>
<tr>
<th>Induction (d)</th>
<th>IgM Secretion (ng/mL)</th>
<th>[3H]-ThdR Incorporation (cpm x 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninduced</td>
<td>24 ± 2</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>TPA</td>
<td>550 ± 63</td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>SAC + BSF-MP6+TNF-α</td>
<td>125 ± 13</td>
<td>74 ± 2.6</td>
</tr>
<tr>
<td>SAC + BSF-MP6+IL-2</td>
<td>400 ± 46</td>
<td>548 ± 29</td>
</tr>
<tr>
<td>TPA + BSF-MP6+IL-4</td>
<td>5,600 ± 510</td>
<td>291 ± 16</td>
</tr>
<tr>
<td>SAC + BSF-MP6+IL-2+IL-4</td>
<td>59 ± 6</td>
<td>21 ± 0.9</td>
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I83 cells were stimulated by the indicated substances for 6 days and analyzed with respect to functional differentiation and proliferation, as described in Materials and Methods and detailed in Larsson et al.40

Figure 3A shows an intense bcl-2 staining in the sparse cytoplasm of untreated I83 cells. Also, cells treated by TPA for 6 days showed an intense cytoplasmatic staining, distributed rather homogeneously within the population (Fig 3B). In contrast, cells stimulated by SAC + BSF-MP6 + IL-2 exhibited a significantly reduced expression of bcl-2, particularly among the Ki-67–positive B blasts, but also apparent in a substantial part of the Ki-67–negative population, usually resembling B blasts or activated lymphocytes (Fig 3C). Although most Ki-67–positive cells exhibited a low bcl-2 expression, occasional cells, usually with plasmablast morphology, expressed both Ki-67 and bcl-2 at a high level (Fig 3C). After TPA + BSF-MP6 + IL-4 inductions, which result in a pronounced morphologic and functional differentiation of I83 cells, a more complex distribution of bcl-2 and Ki-67 expression was found. As in SAC + BSF-MP6 + IL-2–stimulated cells, bcl-2 was expressed in the small- to medium-sized lymphocytes and generally had low expression in the larger Ki-67–positive B blasts, whereas the expression in middle-sized lymphocytes was more heterogeneous. However, bcl-2 staining was also often intense in the more differentiated TPA + BSF-MP6 + IL-4–induced cells, resembling plasmablasts (Fig 3D). Corinna II cells, representing EBV-immortalized CLL cells, showed a very weak bcl-2 staining (Fig 3E).

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In agreement with recently reported data,38,41 all B-CLL cases expressed bcl-2. The level of expression seems unrelated to the clinical stage of the patients and does not reflect a rearrangement of bcl-2, which has been shown to be a rare event.42 The fact that the bcl-2 gene is translocated into the Ig light chain loci in approximately 10% of CLL cases studied10 and that B-CLL cells express a higher level of
bcl-2 than normal CD5+ B cells (Fig 1) might suggest an inappropriate malignancy-related expression of bel-2 in B-CLL. One may thus interpret these results as an inability of the malignant B-CLL cells to switch off the bcl-2 expression, even in the absence of classical rearrangements, and hypothesize that bcl-2 expression, perhaps by inhibiting apoptosis, could confer to B-CLL cells a survival advantage that favors their relentless accumulation.

However, we have shown that in two selected clones of B-CLL the expression of bcl-2 is not constitutive, but can be modulated. bcl-2 is rapidly but transiently downregulated in both I73 and I83 B-CLL clones exposed to TPA (Fig 1), which leads CLL cells to enter a differentiation pathway that proceeds in the absence of DNA synthesis. Kinetically, this reduction parallels the rapid downregulation of µ-chain mRNA and the increase in c-myc RNA previously reported.39 In contrast, the reduction of levels of bcl-2 mRNA and protein appears to be sustained and not transient after the stimulation of I83 cells in response to SAC + BSF-MP6 + IL-2 and TPA + BSF-MP6 + IL-4 (Figs 1 and 2). These two induction protocols stimulate the highest level of DNA synthesis and, thus, the results indicate that proliferation and bcl-2 expression are inversely related in these B-CLL cells. Two further observations support this interpretation. First, in the I83 B-CLL clone, the immunocytochemical analysis of individual cells shows that bcl-2 is primarily expressed in resting small lymphocytes and, after induction, in cells at the plasmablast-plasma cell stages, while it is
much less expressed in the B blasts (Fig 3), which comprise most of the Ki-67-positive, proliferating cell elements at an intermediate stage of B-cell differentiation. This finding is in agreement with our recent observation that bcl-2 is expressed in normal plasma cells of the bone marrow and the lymph node medulla and in human myeloma cell lines. However, in contrast to Pezzella et al., we have found occasional cells that coexpress bcl-2 and Ki-67 at a high level (Fig 3). These were usually morphologically more mature than B blasts and it is presently unclear whether they still progress into the cell cycle. Second, the bcl-2 level observed in the exponentially growing lymphoblastoid cell line (LCL) and B-CLL cell line Corinna I and II is low (Fig 2). In SAC + BSF-MP6 + IL-2 and TPA + BSF-MP6 + IL-4-treated CLL cells the small- to middle-sized, usually Ki-67-negative cells that morphologically resemble activated lymphocytes often exhibit low bcl-2 expression. This contrasts with the behavior of cells of similar morphology in TPA-treated cultures, which uniformly show higher levels of bcl-2. We speculate that B cells determined to traverse the cell cycle into S-phase, rather than to enter a differentiation pathway in the absence of proliferation, will downregulate bcl-2 at an early stage, before becoming Ki-67 positive. Downregulation of bcl-2 expression in B cells could thus be related to the choice of entering a self-renewal pathway and mirror the myc regulation operating when the cell choice is toward the differentiation pathway.

The reduced expression of bcl-2 in B-CLL after mitogenic stimulation contrasts with the increased expression observed in normal tonsillar B cells and PBL (unpublished results). This may be interpreted as aberrant bcl-2 regulation in B-CLL associated with the malignant phenotype, as discussed above. An alternative explanation is that both the high level of expression of bcl-2 in untreated B-CLL cells and the differential regulation of bcl-2 in response to stimuli reflect a normal regulation occurring in a subtype of B cells and/or a particular stage of B-cell development and may thus help in identifying the cellular origin of B-CLL. Our results showing that normal resting follicular mantle zone B cells express bcl-2 mRNA and protein that is lost in actively proliferating follicular germinal center B cells agree with recent reports and show that a similar pattern of bcl-2 expression as in B-CLL cells occurs in certain normal B cells. Therefore, it becomes pertinent to ask which are these normal B cells and to analyze whether there are other similarities between follicular mantle cells and resting B-CLL cells and between GC cells and in vitro activated B-CLL cells. Mantle B cells include a subpopulation of long-lived recirculating B cells that express sIgD and CD23, a feature also found with B-CLL. Based on the long lifespan of B-CLL cells and their surface antigen expression, it has previously been suggested that B-CLL cells might represent the malignant counterparts of long-lived recirculating B cells. Based on CD5 antigen expression, B-CLL cells have been regarded as the malignant equivalents of normal CD5+ B lymphocytes. These two interpretations do not conflict because, in adult lymphoid tissues, CD5+ B cells appear to be located within the mantle zone of secondary follicles. Furthermore, stimulated CD5+ B cells (both normal and malignant) acquire similarities with GC-B cells both at the phenotypic level and with respect to factor requirement for growth in vitro. Our observation that CD5+ normal B cells from cord blood, which exhibit an activated phenotype, do not express bcl-2 may also support this notion.

On this basis, our observations led us to formulate the hypothesis that the high expression of bcl-2 in B-CLL cells reflects their affiliation to a long-lived memory type of B cell (Fig 5) located in the mantle zone of lymphoid follicles. This hypothesis is well in keeping with the transgenic mouse model, in which it has been shown that bcl-2 influences the generation and maintenance of B-cell memory. It is thus possible that B-CLL may represent a tumor characterized by an abnormal balance in the production of terminally differentiated cells versus memory cells that may be used as a model system for studying memory B cell behavior. Further investigation is required to establish the role of CD5+ normal B cells in this scenario.
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